PE4.37 Time-related changes of Zn-chelatase and Zn-protoporphyrin IX in dry-cured hams 134.00

Tania Toscani (1) tania.toscani@prosciuttodiparma.com, Riccardo Benedini(2), Giovanni Parolari 2,
(1)Consorzio del Prosciutto di Parma
(2)Stazione Sperimentale per l'Industria delle Conserve Alimentari

***Abstract*—Red colour in nitrate-less dried hams is contributed by zinc protoporphyrin IX (ZPP) whose formation occurs at later stages of the curing process. In order to investigate the synthesis reaction as affected by a variety of substances either present in the meat or added as curing ingredients, a study was undertaken on the effect and behaviour of Zn-chelatase, and of commonly used additives such as sodium nitrate and ascorbate. Results suggest that formation of ZPP is encouraged by the enzyme, whose changes over the manufacturing time account for the final colour outcome. Of the two additives, nitrate inhibits the pigment whereas ascorbate enhances its formation, suggesting that colour development in nitrate-less dried meat can be affected by the chemical compounds used as curing adjuncts.**

***Index Terms*: dry cured ham, nitrate-less, colour, zinc-protoporphyrin, Zn-chelatase**

I. INTRODUCTION

Whereas the development of colour in dried hams relies typically on nitrite and/or nitrate, in dried Parma hams a stable red pigment is achieved by the addition of nitrite-less sea salt only [1]. Such pigment has been identified [2-4] as zinc-protoporphyrin IX (ZPP) which would form within the muscle by zinc replacement of the iron in the heme ring. While the mechanism yielding ZPP is still to be clarified, its formation has been supposed to be supported by an endogenous enzyme such as Zn-chelatase, whose activity has been reported in a variety of food substrates including raw meat [5]. However, no study has been focused on the activity of enzyme in the leg's muscles, nor on its changes during the manufacturing time of Parma ham, which is at least of one year.

We have developed an assay procedure for the measurement of ZPP promoting activity in pork meat with the aim to i) classify green legs for their Zn-chelatase activity, ii) elucidate the formation of ZPP as affected by Zn-chelatase in individual muscles at various stages of Parma ham processing, and iii) test several substances, amongst those typically used in the meat industry, for their effects on the enzyme activity.

II. MATERIALS AND METHODS

A. Samples

Raw meat samples (N=138) from green thighs purchased from local manufacturers consisted in a slice of 1 cm thickness from the outer *semimembranosus* muscle. Additional samples (N=96) were obtained from hams at the following processing stages: green hams (prior to salting), end of resting phase (90 days after salting), mid-maturing (6-8 months), end of maturing (12 months) and after extensive ageing (18-20 months). From these hams the following muscles were removed for analysis: *biceps femoris* (BF), *semimembranosus* (SM), outside *semitendinosus* (light ST), inside *semitendinosus* (dark ST) and *rectus femoris* (RF).

In a subsequent experiment aimed at the effect of salt, nitrate and ascorbate on ZPP formation, N=48 raw legs were dry cured with either salt alone, or salt+ascorbate (500 mg/kg meat) or salt+nitrate (200 mg/kg meat). They were then processed according to procedures in use for traditional Italian dried hams and analysed for their ZPP content at several manufacturing times.

B. Zn-chelatase activity assay

Muscle extracts were prepared and assayed for Zn-chelatase according to the method described by Benedini and others [6]. Trimmed muscle (8 grams) was homogenized with ice cold tris-HCl 20 mM, glycerol 20% w/v, KCl 0.8% w/v and Triton® X-100 (Sigma-Aldrich, St.Louis, U.S.A.) 1% w/v. After stirring for 30 min at 4 °C, the homogenate was centrifuged (Avanti J-30I, Beckman coulter) at 15000 × g for 10 min. Extraction was performed twice, final volume was 80 mL. The muscle extract was incubated at 37 °C, pH=7.0 for 45 min in the dark with $ZnSO_4$ 100 □M , protoporphyrin IX (Sigma-Aldrich) 50 µM, NaCl 4% w/v and adenosine 5'-triphosphate dipotassium salt dihydrate (Sigma-Aldrich) 5 mM. Each extract was assayed against a blank obtained adding EDTA (1.75 mM) to the reaction mixture. After

incubation, the enzymatic reaction was stopped by adding EDTA. The content of the tubes was combined 1:1 with ethanol 96% (v/v) and the resulting solution centrifuged at 26000 × g for 10 min. Finally, the clear supernatant was submitted to fluorescence analysis (500-700 nm), with excitation at 415 nm and emission at 590 nm. Each extract was assayed twice.

C. Analysis of zinc-protoporphyrin IX (ZPP)

Pigment extraction was performed according to the method of Wakamatsu and others [2] as adapted by Adamsen and others [7]. The trimmed muscle (10 grams) was homogenized with 100 mL of cold 0.2 M phosphate buffer (pH 6.0) and the slurry centrifuged at 20000 × g, 2 °C for 20 min. The supernatant was filtered through paper and the aqueous extract treated with acetone:water (3:1, v/v) then held on ice for 30 min and filtered through paper. Zinc-protoporphyrin was determined fluorometrically (excitation at 415 nm, emission at 590 nm), with acetone:water (75:25, v/v) as the blank. Emission intensity was used as a measurement of the amount of ZPP in the extract [8-9].

III. RESULTS AND DISCUSSION

A. Classification of raw meat based on Zn-chelatase activity

As shown in fig.1, Zn-chelatase in raw meat encompasses a broad range of values, ranging from values of slightly above zero to as much as 7 units/gram dry matter. Such large differences suggest a specific attitude of individual hams to development of ZPP which might result in large inconsistencies in finished ham colour. It is to be noted that the enzyme assay in this part of the study was limited to an external muscle such as the *semimembranosus*, with no additional information on the other leg's muscles, namely the inner ones. Therefore a subsequent investigation was targeted to the relationships between enzyme activities in a variety of leg's muscles and their changes at various processing times.

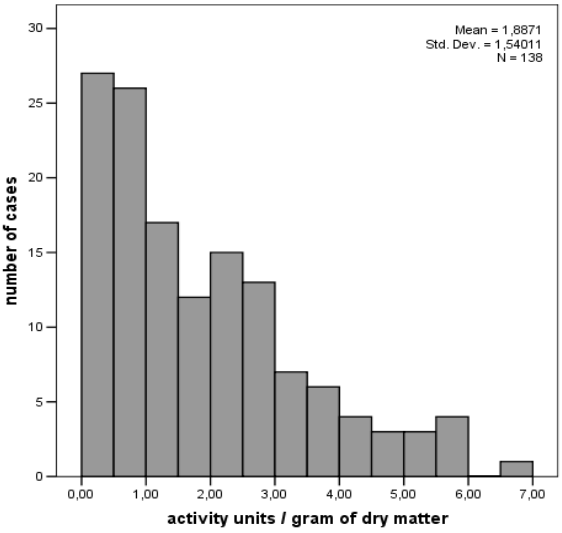

Figure 1. Distribution of Zn-chelatase activity levels, in units/gram of dry matter, in N=138 samples of outer *semimembranosus* muscle.

B. Zn-chelatase activity in green legs and cured ham's muscles

Zn-chelatase activities are reported in Fig.2A for each of the five muscles at several processing times. In raw thighs, major differences between muscles are found within the same leg, with dark ST significantly greater ($P<0.05$) than light ST. At successive stages enzyme activities in the BF are generally greater than in other muscles, with RF and light ST exhibiting lower values. Therefore the broad range of activities found in raw muscles indicate that Zn-chelatase is strictly dependent upon individual muscles even if they belong to the same primal cut [10].

This study was also aimed at the behaviour of the enzyme as affected by chemical changes occurring in the meat during maturation. Results in fig.2A show that there is considerable enzyme activity even after 18-20 months of ageing, with average values of 0.88 units/gram dried meat. This means that the meat has a potential to yield ZPP even at the latest steps of manufacturing. This observation is in agreement with the increased ($P<0.05$) fluorescence (or ZPP) measured during the last stage of processing (ageing), when leg's muscles increase from 263.2 to 330.8 (Fig.2B).

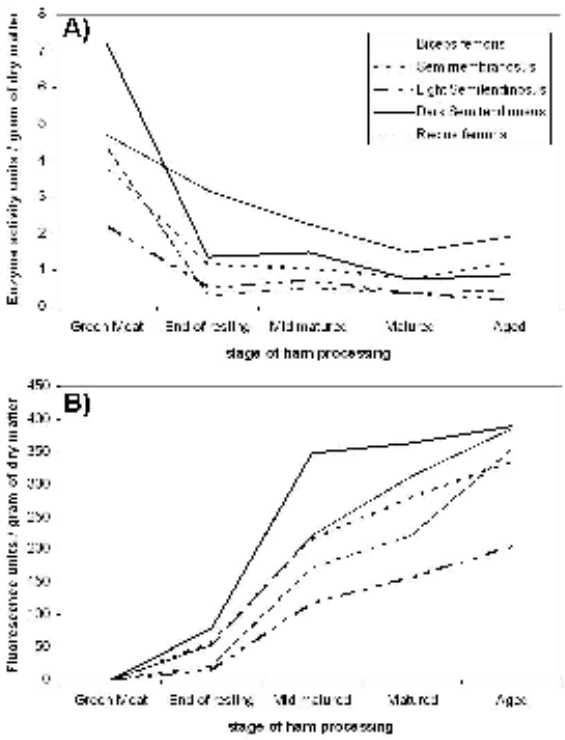

Figure 2. Time-related changes of A) Zinc-chelatase activity (units/gram dry matter) and B) Fluorescence emission intensity (fluorescence units/gram dry matter).

As shown in Fig 2A, the enzyme activity drops dramatically in the first three cold resting months, with an additional slight decrease over the remaining manufacturing time. This trend is not exactly the same for each muscle, since dark ST loses a total of 88% of its original activity, while BF and SM decrease 59% and 68% respectively, keeping greater residual activity.

As a results, fluorescence values achieved in finished BF and SM (Fig.2B) are very similar to those developed by dark ST. Of the two remaining muscles (light ST and RF), both low in enzyme activities, the former exhibits less fluorescence whereas the latter displays eventually the same fluorescence as the average ham. Therefore, time-related changes in enzyme activity are supportive of a muscle-specific pathway to ZPP formation.

It is noteworthy that ZPP forms late in the manufacturing time (fig. 2B), as shown by the increase of fluorescence occurring only after the accomplishment of the resting stage or after 3 months of curing. This means the pigment could not form until the temperature was kept at 1-3°C (salting and resting phases), while it increased sharply when the hams when allowed to mature at 15-18°C. This finding is supportive of an enzyme-based reaction leading to zinc-protoporphyrin, where the enzyme needs mild temperatures to be activated.

C. Zn-chelatase activity as affected by curing agents

The ZPP-promoting activity in the muscle extract was assayed in the presence of substances which are commonly used as ingredients or additives in dry cured meats. Results, reported (Table 1) in activity units vs. concentration of the added compounds, indicate that salt in the range 0-80 mg/l enhances the enzyme, while sodium nitrate has no effect. Sodium ascorbate promotes the enzyme when at 500 mg/l, whereas glucose has no influence at any of the concentrations tested (0-1000 mg/l). Finally, pH changes over the range 5.5-7.5 were investigated, resulting in apparent decrease of activity at augmented pH values.

D. Effect of curing-agents on ZPP formation in dry cured hams.

The effect of curing agents selected amongst those commonly used in dry curing of hams was investigated by treating raw legs with either sea salt (no additives added) or sea salt+ascorbate (500 mg/kg meat) or sea salt+nitrate (200 mg/kg meat). Data, graphically reported in fig. 3, demonstrate that ascorbate plays a positive role on the synthesis of ZPP [11], in accordance with previously shown results from model system testing (tab. 1). In contrast, nitrate-added hams exhibit lower pigment content ($P<0.05$) at later processing stages compared with both their nitrate- and salt-treated counterparts. This finding fails to match model system observations (tab. 1) where nitrate was found ineffective. One reason might be that nitrosyl oxide, which is reportedly the key molecule in ZPP inhibition [12] can be delivered by nitrate in real hams but not in a short-terming model system.

Table 1. Zn-chelatase (activity units/gram dry matter) in fresh meat extracts added with curing agents. Mean values of 4 replicate analyses.

Curing agents	Concentration in the extract	Zn-Chelatase *activity units* (st. dev.)
Sodium Chloride	0 g/l	0.60 (0.12)[a*]
	20 g/l	1.34 (0.13)[b]
	40 g/l	2.00 (0.23)[c]
	60 g/l	2.60 (0.26)[d]
	80 g/l	3.04 (0.21)[e]
Sodium Nitrate	0 mg/l	2.05 (0.13)
	50 mg/l	2.13 (0.26)
	150 mgl	1.99 (0.24)
	300 mg/l	1.92 (0.18)
Sodium Ascorbate	0 mg/l	2.13 (0.25)[a]
	100 mg/l	2.39 (0.25)[a,b]
	300 mg/l	2.42 (0.30)[a,b]
	500 mg/l	2.57 (0.30)[b]
Glucose	0 mg/l	1.03 (0.04)
	200 mg/l	0.99 (0.08)
	500 mg/l	1.03 (0.12)
	1000 mg/l	0.86 (0.10)
pH	5.5	1.37(0.14)[a,b]
	6.0	1.49 (0.14)[a]
	6.5	1.14(0.01)[b]
	7.5	1.06 (0.01)[c]

[*] for each substance, values with different superscript letters are significantly different (P<0.05).

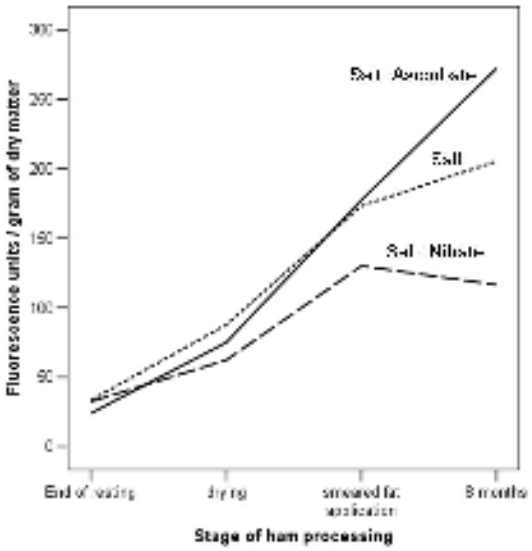

Figure 3. Time-related changes of ZPP (fluorescence units/gram dry matter) in hams added with either salt, or salt+ascorbate, or salt+nitrate**.**

IV. CONCLUSION

Results from this research indicate that Zn-protoporphyrin (ZPP) is promoted by Zn-chelatase acticity in the muscle. Changes in the enzyme activity during processing account for final colour outcome, with hams exhibiting better colour consistency in dried than in green muscles. The combined effect of the enzyme and of an external additive such as ascorbate would deserve further investigation in order to enhance the formation hence redness of nitrate-less dried pork meat.

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